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# Profilin isoforms in *Dictyostelium discoideum*

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**Abstract**

Eukaryotic cells contain a large number of actin binding proteins of different functions, locations and concentrations. They bind either to monomeric actin (G-actin) or to actin filaments (F-actin) and thus regulate the dynamic rearrangement of the actin cytoskeleton. The *Dictyostelium discoideum* genome harbors representatives of all G-actin binding proteins including actobindin, twinfilin, and profilin. A phylogenetic analysis of all profilins suggests that two distinguishable groups emerged very early in evolution and comprise either vertebrate and viral profilins or profilins from all other organisms. The newly discovered profilin III isoform in *D. discoideum* shows all functions that are typical for a profilin. However, the concentration of the third isoform in wild type cells reaches only about 0.5% of total profilin. In a yeast-2-hybrid assay profilin III was found to bind specifically to the proline-rich region of the cytoskeleton-associated vasodilator-stimulated phosphoprotein (VASP). Immunolocalization studies showed similar to VASP the profilin III isoform in filopodia and an enrichment at their tips. Cells lacking the profilin III isoform show defects in cell motility during chemotaxis. The low abundance and the specific interaction with VASP argue against a significant actin sequestering function of the profilin III isoform.


*Keywords:* Actin monomer binding proteins; Profilin I/II; Phylogeny; Biochemical analysis; Mutant analysis

## 1. Introduction

*Dictyostelium discoideum* is a free living soil amoeba with an elaborate cytoskeleton, which has made it a model system of choice to study cytoskeleton based processes. When starved, *D. discoideum* cells initiate a developmental programme where cAMP acts as a chemoattractant leading to the formation of multicellular aggregates which organize themselves to form a tipped aggregate, then a slug which exhibits phototaxis and thermotaxis, and finally to culminate into a fruiting body by 24 h after the onset of starvation (for reviews see [1,2]). All stages of the life cycle are characterized by and depend on the chemotactic motility of the individual cells, and the remodeling of their actin cytoskeleton is an essential aspect of their life [3,4]. The dynamic rearrangements are controlled by a large number of actin-binding proteins which have different functions and can be grouped into different families. The majority of them interact with filamentous actin (F-actin) either by capping the ends, by severing filaments into smaller fragments, by binding to the sides thus bundling or crosslinking filaments into three-dimensional networks, by anchoring filaments to membranes or vesicles, and last but not least by using motor proteins like myosins along actin filaments to transduce mechanical force. A much smaller number of proteins has been characterized as binding to globular actin monomers (G-actin). The intrinsic problem for these proteins is the enormous concentration of actin in a cell and, consequently, the need for high concentrations of monomer binding protein if it has to accomplish a change in the G- to F-actin equilibrium.

Monomer binding and sequestering proteins as classified in Eichinger et al. [5] are mainly profilins, CAP (cyclase-

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associated protein), actobindin-like, twinfilin-like and in general WH2-containing proteins. They are evolutionarily conserved and have been found in organisms from yeast to animals. Profilin and CAP are also present in plants. The interaction of the monomer binding proteins with actin appears to be based on the presence of a limited number of protein motifs. Although these motifs are structurally different, their binding sites on actin subdomains 1 and 3 partially overlap (reviewed in Paavilainen et al. [6]).

*D. discoideum* harbors representatives of all classes of actin monomer binding proteins, actobindins, CAP, WH2-containing proteins, twinfilins and profilins. Actobindin was first isolated from *Acanthamoeba castellani* [7,8]. It inhibits actin polymerization by sequestering G-actin and stabilizing actin dimers. Its homolog in mammals is β-thymosin which in vivo builds a reservoir of unassembled actin by forming a 1:1 complex with ATP-G-actin [9]. Actobindin consists of two β-thymosin repeats [19], the homologous proteins in *Caenorhabditis elegans* and *Drosophila melanogaster* consist of four and three thymosin repeats, respectively [10–12]. The *D. discoideum* genome harbors three actobindin genes, *abnA*, *B* and *C* which code for small proteins consisting of two thymosin repeats. The products of *abnB* and *C* are identical and share 72% identity with the one of *abnA*. Actobindins are also present in *Entamoeba histolytica* and share about 60% similarity with the *D. discoideum* proteins.

CAP/Srv2 (cyclase-associated protein/suppressor of Ras-Val19) is a highly conserved and widely distributed protein with roles in normal cell growth and regulation of the cytoskeleton (reviewed in [13]). It was first identified in *Saccharomyces cerevisiae* where it is required for the activation of adenylyl cyclase by Ras [14,15] and for the correct functioning of the cytoskeleton. CAP homologs have been identified in a variety of organisms including yeast (*S. cerevisiae*, *Schizosaccharomyces pombe*), mammals (human, rat, mouse), amoebae (*D. discoideum*), plants (maize, *Arabidopsis thaliana*) and *Drosophila*. CAP harbors also a WH2 domain which may act as an actin-binding region, although experimental data have not been provided [16]. Higher eukaryotes have two or more CAPs that are highly related, whereas lower eukaryotes and many plants have only a single CAP. *D. discoideum* has five more WH2-containing proteins that have been classified as actin–monomer binding proteins. They are unique to *D. discoideum* and no biochemical data are available on their interaction with actin so far.

Twinfilins are composed of two actin depolymerizing factor homology (ADF-H) domains [17]. They are involved in diverse morphological and motile processes and have been proposed to sequester ADP-G-actin and cap filament barbed ends [18]. In a very recent report purified budding yeast twinfilin was shown to bind to and sever actin filaments in vitro at a pH below 6.0 [19]. Phylogenetic comparisons reveal distinct differences between twinfilins from lower eukaryotes, whereas in higher eukaryotes twinfilins are very well conserved. *D. discoideum* has a single twinfilin.

Profilin is an essential protein with cellular functions related to the actin cytoskeleton, including motility, development, signaling and membrane trafficking. The diversity of its functions might be due to the many interactions of profilin which extend to phosphoinositides and proline-rich domains in proteins [20,21]. Profilins are now considered to function not only in depolymerization but also in polymerization of F-actin [22].

Here we focus on the profilins from *D. discoideum*. The *D. discoideum* genome encodes three profilins [5]. Profilins I and II which have been characterised in detail previously are both abundant proteins which efficiently sequester G-actin [23,24]. Mutants lacking a single isoform showed an essentially unchanged phenotype, whereas double mutants had increased cortical actin, reduced motility and a cytokinesis defect [23]. Profilin III was only discovered recently and its characterisation and in vivo role are reported here.

## 2. Materials and methods

### 2.1. Strains and growth conditions

*D. discoideum* strain AX2 and transformants were cultivated in nutrient medium in submerged or shaking cultures (150 rpm) at 21 °C essentially as described [25] using HL5 medium mainly for transformants and AX2 medium for routine axenic cultures (14.3 g/l bacteriological peptone, 7.15 g/l yeast extract, 18 g/l glucose, 4 mM $Na_2HPO_4$, 3.6 mM $KH_2PO_4$, pH 6.7). For development of *D. discoideum*, cells were grown to a density of $3 \times 10^6$ cells/ml, washed in phosphate buffer (14.6 mM $KH_2PO_4$, 2 mM $Na_2HPO_4$, pH 6.0), deposited ($10^8$ cells/plate) on phosphate agar, allowed to develop for the appropriate time, and then harvested for preparation of protein or RNA.

### 2.2. Molecular cloning, expression and purification of recombinant profilin III

For the expression of recombinant profilin III, the entire coding region of the *proC* gene was amplified from AX2 genomic DNA by PCR as a 381-bp *Nde*I/*Xba*I fragment using the specific primers 5′ GCGCA TATGA CTTGG CAAGC ATATA TGATAC 3′ and 5′ GCGTC TAGAT TAGAA ACCTT GTTCT CTTAA ATAATC 3′, cloned into the expression vector pT7-7, and verified by DNA sequencing. After transformation of the *E. coli* strain BL21 the cells were grown at 37 °C at 220 rpm and expression induced with 1 mM IPTG at an $O.D._{600}$ of about 0.6. Profilins I and II were expressed in the *E. coli* strain JM83, the purification of all three isoforms followed the same procedure. After 5–6 h at 37 °C post induction cells were harvested, washed, resuspended in homogenization buffer (10 mM Tris/HCl, 1 mM EGTA, 1 mM DTT, 0.02% sodium azide, 5 mM benzamidine and 0.5 mM PMSF, pH 8.0), and opened as described [26]. The recombinant protein was in inclusion bodies and stepwise extracted with increasing concentrations of urea. Most of the protein was solubilized in 5 M urea. The protein extract was then slowly dialyzed against homogenization buffer, bound to a poly L-proline affinity resin and eluted essentially as described [24]. Recombinant profilin I and II have been cloned into the pIMS expression vector essentially as described [27]. None of the profilins contained a tag.

### 2.3. Inactivation of the proC gene, identification of knockout mutants and isolation of overexpressing mutants

For construction of the proC gene replacement vector, a 400-bp *Bam*HI/*Pst*I fragment of the 5′ end upstream of the coding region and a 600-bp *Hin*dIII/*Sal*I fragment downstream were amplified from genomic AX2 DNA. Both fragments were cloned into the corresponding sites of pLPBLP containing the blasticidin resistance cassette [28]. The resulting vector was cleaved with *Bam*HI and *Sal*I and used to replace the proC gene in wild type cells. The integration of the gene replacement cassette was analyzed by PCR and in an immunoblot for the absence of profilin III. Profilin III rescue mutants were generated by expressing full length profilin III that was fused to a Flag peptide at its N-terminus.

### 2.4. Actin polymerization assays

Actin polymerization was measured by fluorescence spectroscopy using a LS55 fluorometer (Perkin Elmer, Rodgau, Germany) with pyrene-labeled actin as described previously [29]. To compare the affinities of all three profilin isoforms to actin, the proteins were purified to homogeneity, mixed at the appropriate molar ratios with G-actin and incubated for 2 min at room temperature. Actin polymerization was started by addition of 10 mM imidazole, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM Na-ATP (pH 7.2). Care was taken that all profilin isoforms during preincubation with G-actin had the same time to form 1:1 profilin:actin complexes. The correspondingly reduced concentrations of free G-actin extended the nucleation of polymerization and lowered the slopes of the linear part of the elongation phase. The slopes were used for calculation of the Kd values and reflect mainly the stability of the profilin:actin complexes. Even at extremely high profilin concentrations, however, the elongation could not be reduced below about 10% of the controls with actin alone. This may reflect the resultant of two antagonistic phenomena: sequestration of actin monomers by profilin and shuttling of actin as profilin–actin complex to the barbed filament ends. This latter activity was never a major factor in reaching saturation of elongation and we assume that the calculated Kd values represent almost entirely the sequestering function of the profilin isoforms.

### 2.5. Interaction of $PIP_2$ with profilin III

For cosedimentation of recombinant profilin III with $PIP_2$ the protein was dialyzed against IEDANBP buffer (10 mM imidazole, 1 mM EGTA, 1 mM DTT, 0.02% $NaN_3$, 100 mM NaCl, 1 mM benzamidine, 0.5 mM PMSF, pH 7.6), incubated with $PIP_2$ vesicles (Sigma) for 5 min on ice in a total volume of 150 µl, and centrifuged for 20 min at 100,000×*g*. The resulting supernatants and pellets were mixed with SDS sample buffer and analyzed by SDS-PAGE.

Gel filtration of profilin III in the presence or absence of $PIP_2$ was performed on a Smart System (Pharmacia) in IEDANBP buffer using a Superose 12 column. The column was calibrated with a commercially available mixture of marker proteins. The eluted fractions were analyzed by SDS-PAGE and Coomassie blue staining.

### 2.6. Yeast-two-hybrid assay

The activation domain (AD) of pGADT7 was fused to full length DdVASP (*vasP*, DDB0229340, 1–380) and to the domains EVH1 (1–113), PRD (114–192), EVH2 (193–380), EVH1-PRD (1–192), and PRD-EVH2 (114–380), and the respective DNA constructs cloned as *Bam*HI and *Sal*I fragments. All three profilin isoforms were fused with the Gal4-binding domain (BD) in plasmid pGBKT7 as *Eco*RI and *Sal*I fragments. Yeast cells were transformed with an appropriate plasmid pair, were plated onto Leu-/Trp- plates to isolate cotransformants, and subsequently replica plated onto Leu-/Trp-/His- plates to test the interaction.

### 2.7. Immunofluorescence microscopy

Analysis of profilin III localization was performed via indirect immunofluorescence. Cells were fixed with paraformaldehyde/picric acid solution (2% paraformaldehyde, 10 mM Pipes, 15% saturated picric acid, pH 6.0) essentially as described [30]. Profilin III was detected using affinity purified polyclonal antibodies and Cy3-labelled goat anti mouse IgG secondary antibodies (Dianova, Germany). Cells were scanned at 0.05 µm distance intervals along the *Z*-axis using a LSM510 laser scanning microscope (Carl Zeiss, Germany), and 3D-reconstructions of the optical sections were done with the Axiovision software as described previously [31]. To quantify the nuclei cells were fixed with ice-cold methanol for 10 min at −20 °C followed by 30 min air drying. Nuclei were stained with DAPI (4, 6-diamidino-2-phenylindole, Sigma).

### 2.8. Analysis of cell shape and cell migration

Aggregation competent wild type and mutant cells were seeded onto glass coverslips in small plastic dishes, and cell migration towards a microcapillary filled with $10^{-4}$ M cAMP in a micromanipulator system (Eppendorf, Germany) was recorded at intervals of 10 s on an Axiovert200 inverted microscope (Carl Zeiss, Germany) essentially as described [32]. Speed and orientation were analyzed with the 2D DIAS programme (Solltech, USA) as summarized in Wessels et al. [33]. For a detailed shape analysis, the outlines of the individual cells were drawn manually.

### 2.9. Quantitative PCR/real-Time RT-PCR

Total RNA was extracted from growing AX2 cells and cDNA prepared using the M-MLV reverse transcriptase, RNAse H minus (Roche, Switzerland) according to the manufacturer's protocol. Total RNA was used for the RT reaction. For quantitative PCR analysis of profilin I, II and III, specific primers were selected such that the expected product size was between 250 and 300 bp. For primer design we used the program http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. The following primers were used: for profilin I forward 5′ AGCTGGCAACAATATGTCGATGAA 3′, reverse 5′ ACTCCACCAATCAAAGCACCCTTA 3′; for profilin II 5′ TGAAGGTAAGGCAATAACCGCACT 3′, reverse 5′ GTTTTAACGCAAACAACACCACCA 3′; for profilin III 5′ CCACTGAAGCCCAACATATCCTTT 3′, reverse 5′ CTTCACAAGCATTAGCAGCAGCAC 3′. Prior to use in real time experiments the quality of the cDNA and the primers were tested by PCR. Real time RT-PCR was carried out with the QuantiTect SYBR green PCR kit (Qiagen, Germany) according to the manufacturer's protocol. For each sample gene specific primers (10 pmol) and 1 µl of cDNA were used. As a quantification standard defined concentrations (25 ng, 2.5 ng, 250 pg and 25 pg) of profilin I cDNA in pUC19 were used. The experiments were carried out using an Opticon Real-Time PCR machine.

### 2.10. Miscellaneous methods

Polyclonal antibodies against *D. discoideum* profilin III were obtained by immunization of one rabbit with recombinant protein, the antiserum was affinity purified on an Affigel 10 matrix coupled with recombinant profilin III, and immunoblotting was performed following routine procedures. Protein concentration was determined according to the method of Bradford [34]. For the quantification of profilin III, the intensities of immunoblot signals were measured using Image J software. Based on the intensities the protein concentration per cell was calculated essentially as described [23]. RT-PCR was done using the Qiagen One-Step RT-PCR kit and gene specific primers.

For the phylograms 121 profilin sequences from the SwissProt database were used. The accession numbers are listed below. The sequences were aligned using the program ClustalW [35], the edges and gaps were trimmed. Trees were created with the protein likelihood program PROML. Tree graphics were made using the Interactive Tree Of Life (ITOL) server and further enhancements to the graphics were made with Adobe Photoshop.

Sequences with the following accession numbers were used: PRO1A_ACACA (P68696), PRO1B_ACACA (Q95VF7) *A. castellanii*, PROF1_AMBAR (Q64LH1) *Ambrosia artemisiifolia*, PROF1_ARATH (Q42449) *A. thaliana*, PROF1_ARTVU (Q8H2C9) *Artemisia vulgaris*, PROF1_BOVIN (P02584) *Bos taurus*, PROF1_CAEEL (Q9XW16) *C. elegans*, PROF1_DICDI (P26199) *D. discoideum*, PROF1_HEVBR (O65812) *Hevea brasiliensis*, PROF1_HORVU (P52184) *Hordeum vulgare*, PROF1_HUMAN (P07737) *Homo sapiens* (Human), PROF1_LILLO (Q9SNW7) *Lilium longiflorum* (Trumpet lily), PROF1_LYCES (Q41344) *Lycopersicon esculentum* (Tomato), PROF1_MAIZE (P35081) *Zea mays* (Maize), PROF1_MALDO (Q9XF40) *Malus domesticus* (Apple), PROF1_MOUSE (P62962) *Mus musculus* (Mouse), PROF1_OLEEU (O24169) *Olea europaea* (Common olive), PROF1_PARJU (Q9XG85) *Parietaria judaica* (spreading pellitory), PROF1_PHAVU (P49231) *Phaseolus vulgaris* (Kidney bean), PROF1_PHLPR (P35079) *Phleum pratense* (Common timothy), PROF1_PHYPO (P22271) *Physarum polycephalum* (Slime mold), PROF1_RAT (P62963) *Rattus norvegicus* (Rat), PROF1_RICCO (O82572) *Ricinus communis* (Castor bean), PROF1_SOYBN (O65809) Soybean, PROF1_STRPU (P32006) *Strongylocentrotus purpuratus* (Purple sea urchin), PROF1_TOBAC (P41372) *Nicotiana tabacum* (Common tobacco), PROF1_WHEAT (P49232) *Triticum aestivum* (wheat), PROF2_ACACA (P19984) *A. castellanii*, PROF2_AMBAR (Q64LH2) *Ambrosia artemisiifolia* (Short ragweed), PROF2_ARATH (Q42418) *A. thaliana* (Mouse-ear cress), PROF2_ARTVU (Q8H2C8) *Artemisia vulgaris* (Mugwort), PROF2_BOVIN (Q09430) *B. taurus* (Bovine), PROF2_CAEEL (Q20025) *C. elegans*,

PROF2_DICDI (P26200) *D. discoideum*, PROF2_HEVBR (Q9STB6) *Hevea brasiliensis* (Para rubber tree), PROF2_HUMAN (P35080) *Homo sapiens* (Human), PROF2_LILLO (Q9SNW6) *Lilium longiflorum* (Trumpet lily), PROF2_LYCES (Q93YG7) *Lycopersicon esculentum* (Tomato), PROF2_MAIZE (P35082) *Zea mays* (Maize), PROF2_MALDO (Q9XF41) *Malus domestica* (Apple), PROF2_MOUSE (Q9JJV2) *Mus musculus* (Mouse), PROF2_OLEEU (O24170) *Olea europaea* (Common olive), PROF2_PARJU (Q9T0M8) *Parietaria judaica* (spreading pellitory), PROF2_PHLPR (O24650) *Phleum pratense* (Common timothy), PROF2_PHYPO (P18322) *P. polycephalum* (Slime mold), PROF2_PONPY (Q5R4E2) *Pongo pygmaeus* (Orangutan), PROF2_RAT (Q9EPC6) *Rattus norvegicus* (Rat), PROF2_SOYBN (O65810) *Glycine max* (Soybean), PROF2_TOBAC (Q9ST99) *Nicotiana tabacum* (Common tobacco), PROF2_WHEAT (P49233) *Triticum aestivum* (Wheat), PROF3_AMBAR (Q64LH0) *Ambrosia artemisiifolia* (Short ragweed), PROF3_ARATH (Q38904) *A. thaliana* (Mouse-ear cress). PROF3_ARATH (Q38904) *A. thaliana* (Mouse-ear cress), PROF3_CAEEL (Q21193) *C. elegans*, PROF3_DICDI (Q8T8M2) *D. discoideum*, PROF3_HEVBR (Q9M7N0) *Hevea brasiliensis* (Para rubber tree), PROF3_HUMAN (P60673) *Homo sapiens* (Human), PROF3_LILLO (Q9SNW5) *Lilium longiflorum* (Trumpet lily), PROF3_ MAIZE (P35083) *Zea mays* (Maize), PROF3_MALDO (Q9XF42) *Malus domesticus* (Apple), PROF3_MOUSE (Q9DAD6) *Mus musculus* (Mouse), PROF3_OLEEU (O24171) *Olea europaea* (Common olive), PROF3_PHLPR (O24282) *Phleum pratense* (Common timothy), PROF3_TOBAC (Q9ST98) *Nicotiana tabacum* (Common tobacco), PROF3_WHEAT (P49234) *Triticum aestivum* (Wheat), PROF4_ARATH (Q38905) *A. thaliana* (Mouse-ear cress), PROF4_BOVIN (Q2NKT1) *B. taurus* (Bovine), PROF4_-HEVBR (Q9M7M9) *Hevea brasiliensis* (Para rubber tree), PROF4_HUMAN (Q8NHR9) *Homo sapiens* (Human), PROF4_MAIZE (O22655) *Zea mays* (Maize), PROF4_ MOUSE (Q9D6I3) *Mus musculus* (Mouse), PROF5_ARATH (Q9FE63) *A. thaliana* (Mouse-ear cress), PROF5_HEVBR (Q9M7M8) *Hevea brasiliensis* (Para rubber tree), PROF5_MAIZE (Q9FR39) *Zea mays* (Maize), PROF6_ HEVBR (Q9LEI8) *Hevea brasiliensis* (Para rubber tree), PROFA_ORYSA (Q9FUD1) *Oryza sativa* (Rice), PROF_ANACO (Q94JN2) *Ananas comosus* (Pineapple), PROF_ANTCR (P18320) *Anthocidaris crassispina* (Sea urchin), PROF_APIGR (Q9XF37) *Apium graveolens* (Celery), PROF_APIME (Q6QEJ7) *Apis mellifera* (Honeybee), PROF_ARAHY (Q9SQI9) *Arachis hypogaea* (Peanut), PROF_BETVE (P25816) *Betula verrucosa* (White birch), PROF_BOMMO (Q68HB4) *Bombyx mori* (Silk moth), PROF_BRABE (Q8T938) *Branchiostoma belcheri* (Amphoxius), PROF_BRANA (Q9FUB8) *Brassica napus* (Rape), PROF_CAMPM (Q8V2L6) *Camelpox virus* (strain M-96), PROF_CAMPS (Q775N7) *Camelpox virus* (strain CMS), PROF_CANAL (P53696) *Candida albicans* (Yeast), PROF_CAPAN (Q93YI9) *Capsicum annuum* (Bell pepper), PROF_CHEAL (Q84V37) *Chenopodium album* (Lamb's-quarters), PROF_CLYJA (P18321) *Clypeaster japonicus* (Sand dollar), PROF_CROSA (Q5EF31) *Crocus sativus* (Saffron), PROF_CUCME (Q5FX67) *Cucumis melo* (Muskmelon), PROF_CWPXB (Q77DS0) *Cowpox virus* (strain Brighton Red) (CPV), PROF_CWPXG (Q80DT4) *Cowpox virus* (strain GRI-90/Grishak), PROF_CYNDA (O04725) *Cynodon dactylon* (Bermuda grass), PROF_DAUCA (Q8SAE6) *Daucus carota* (Carrot), PROF_DROME (P25843) *D. melanogaster* (Fruit fly), PROF_ECTVM (Q8JL78) *Ectromelia virus* (strain Moscow) (ECTV), PROF_ENTHI (P49230) *E. histolytica*, PROF_FRAAN (P0C0Y3) *Fragaria ananassa* (Strawberry), PROF_HELAN (O81982) *Helianthus annuus* (Common sunflower), PROF_LITCN (Q941H7) *Litchi chinensis* (Lychee), PROF_MERAN (O49894) *Mercurialis annua* (Annual mercury), PROF_MUSAC (Q94JN3) *Musa acuminata* (Banana), PROF_NAEGR (Q6QNF8) *Naegleria gruberi* (Amoeba), PROF_PRUAV (Q9XF39) *Prunus avium* (Cherry), PROF_PRUDU (Q8GSL5) *Prunus dulcis* (Almond), PROF_ PRUPE (Q8GT39) *Prunus persica* (Peach), PROF_PYRCO (Q9XF38) *Pyrus communis* (pear), PROF_RABPU (Q6RZE1) *Rabbitpox virus* (strain Utrecht) (RPV), PROF_SCHPO (P39825) *S. pombe* (Fission yeast), PROF_SUBDO (Q9U0E6) *Suberites domuncula* (Sponge), PROF_TETPY (P23412) *Tetrahymena pyriformis*, PROF_TRYBB (Q26734) *Trypanosoma brucei brucei*, PROF_VACCA (O57243) *Vaccinia virus* (strain Ankara) (VACV), PROF_ VACCC (P68695) *Vaccinia virus* (strain Copenhagen) (VACV), PROF_VACCT (Q77TH1) *Vaccinia virus* (strain Tian Tan) (VACV), PROF_VACCV (Q76ZN5) *Vaccinia virus* (strain Western

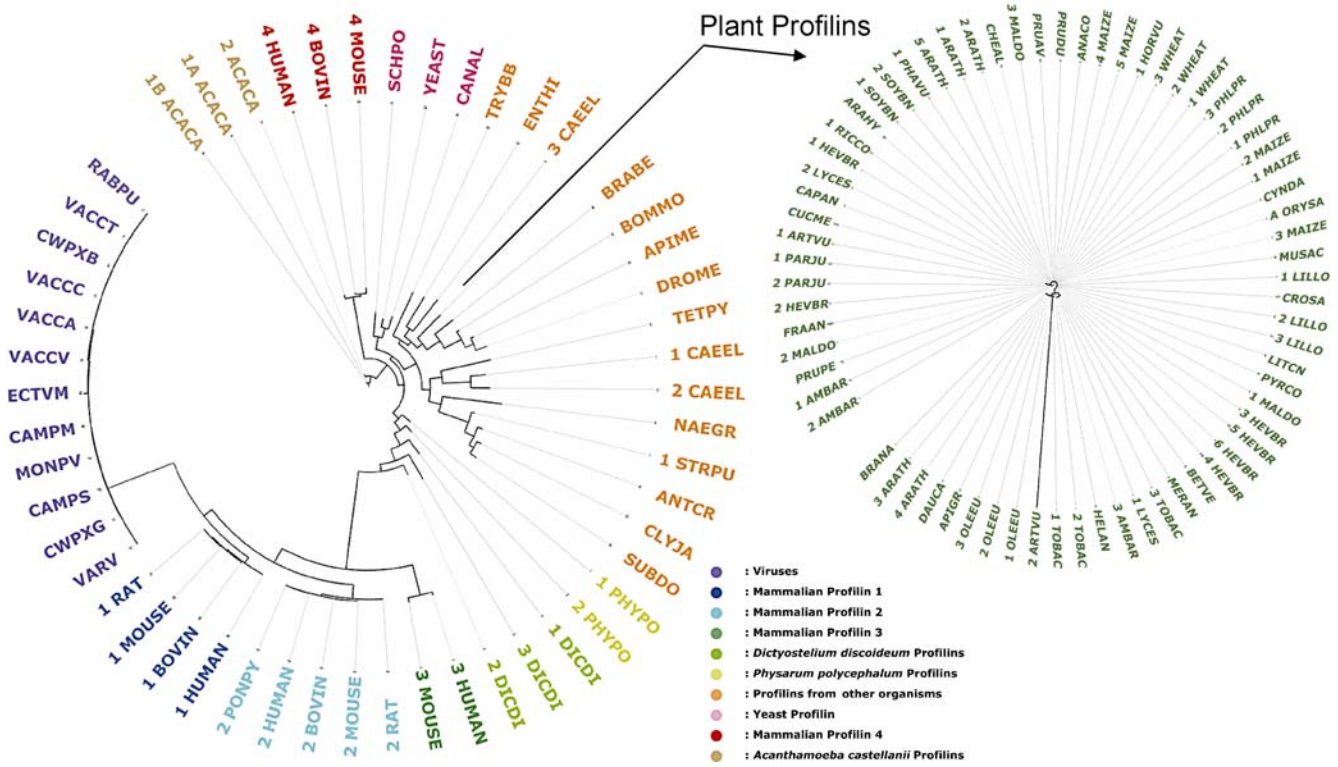



Fig. 1. Evolutionary relationship of profilins. Two data sets of 52 non-plant profilins (left) and 69 plant profilins (right) were analyzed and depicted as two separate radial trees. Plant profilins form a clearly separated and more closely related group of their own, mammalian and viral profilins cluster together as well as all non mammalian profilins which form a separate branch. Mammalian profilins IV are distinct and also belong to this branch. In contrast, the three *D. discoideum* and the two *P. polycephalum* profilin isoforms are closer related to the mammalian and viral profilins than to the majority of other profilins from lower organisms.

Reserve), PROF_VARV (P33828) *Variola virus*, PROF_YEAST (P07274) *S. cerevisiae* (Baker's yeast).

## 3. Results

### 3.1. Phylogenetic analysis of profilins

Profilins are widely present in eukaryotes from plants to animals. More than 50% of all profilin sequences deposited in the databases are plant profilins. Profilins from pollen and from vegetable food are medically relevant as they are frequently allergens [36]. A phylogenetic analysis shows that profilins can be classified into three groups, the plant profilins, which very early separated from the profilin family, the mammalian and viral profilins and the profilins from all other eukaryotes (Fig. 1). Although plant profilins are clearly separated, functionally they seem to be well conserved [37].

Surprisingly, profilin genes have also been identified in the genomes of viruses. They encode proteins that are very similar to vertebrate profilins, however their properties are different. A vaccinia virus profilin which has been analyzed in detail exhibited a low affinity for human platelet actin monomers, had a weak effect on the exchange of the nucleotide bound to actin and no detectable affinity for poly-L-proline, a hallmark of profilins [38]. But it bound to PI4,5P and PI4P and therefore it was proposed that it influences the phosphoinositide metabolism rather than actin assembly. Finally, mutational analysis revealed that this profilin was not essential for viral replication [39]. In a sequence comparison the viral profilins form a class of their own.

Many organisms harbor more than one profilin gene. For mammals recently a fourth profilin gene was reported. Like profilin III, profilin IV is expressed in testis. It is associated with acrosome biogenesis and spermatid elongation [40]. In contrast to mammalian profilin III, profilin IV is more closely related to profilins of lower eukaryotes, protozoa and fungi, which form a separate group, whereas vertebrate profilins I, II and III and the viral profilins cluster together (Fig. 1).

### 3.2. Biochemical functions of the profilin III isoform

Purification and characterization of the two major profilin isoforms (profilin I and II) have been reported more than 10 years ago and there was never any indication that *D. discoideum* might harbor in addition to the genes *proA* and *proB* a third gene that codes for a profilin isoform [23,24]. This gene (*proC*) was found only after the completion of the *D. discoideum* genome [5]. A comparison of all three protein sequences classified profilin III unequivocally as a profilin, sequence identities between pI/pIII and pII/pIII were 44% and 52%, respectively (Fig. 2A). Profilin III contains also at the positions #3 (W), #6 (Y) and #30 (W) residues that have been shown to be required for binding of human platelet profilin to poly-L-proline [41]. As it was reported for *D. discoideum* profilin II, a W3N point mutation abolished binding to a poly-L-proline resin completely [27]. However, *D. discoideum* profilin III contains an alanine residue instead of an usually conserved lysine in position #115 [27] which raised the question whether profilin III has a G-actin binding activity.

Therefore, we thoroughly characterized the third profilin isoform at the biochemical and cell biological levels:

(a) The gene was expressed in *E. coli* BL21 and purified following routine procedures. This included an affinity purification on poly-L-proline beads and elution with 5 M urea (Fig. 2B).
(b) Similar to all other profilins tested, profilin III binds to $PIP_2$. This was assayed in a sedimentation experiment (Fig. 2C) and by chromatography of recombinant profilin III on a gel filtration column in the absence or presence of $PIP_2$ vesicles (Fig. 2D).
(c) Recombinant profilin III inhibited actin polymerization. In a comparison of all three profilin isoforms we calculated Kd values of 5.4 μM, 8.7 μM and 12.9 μM for the recombinant profilins I, II and III, respectively (Fig. 2E). This is in good agreement with the affinities of the profilin isoforms I and II that have been purified from *D. discoideum* cells [24]. Under all conditions the Kd values are in the micromolar range. This renders it as very unlikely that profilin III with its relatively high Kd value and its extremely low cellular concentration is able to sequester G-actin at any significant amount.
(d) Overexpression of the *proC* gene in the pI/II-minus mutants rescued the very strong phenotypes, especially the cytokinesis defect (Fig. 2F) and the block in development just before fruiting body formation (data not shown).
(e) Furthermore, we found a spontaneous reversion of the pI/II-minus phenotype. Detailed analysis of this revertant showed very clearly that the profilin I and II isoforms were still absent, but the profilin III concentration was, by unknown reasons, strongly upregulated and thus could replace at least to a certain extent the missing profilins (data not shown).

In summary, the data show that profilin III exhibits in vitro and, if overexpressed also in vivo all major functions of a normal profilin.

### 3.3. proC is expressed throughout development

Because the aberrant phenotype in the pI/II-minus mutants included defects during growth phase (cytokinesis) and late development (lack of fruiting body formation) we performed RT-PCR experiments on total RNA preparations from wild type cells. The data clearly showed that *proC* is expressed throughout development at roughly equal concentrations (Fig. 3A). This is in agreement with the observations on the spontaneous revertant, i.e. an upregulation of the endogenous gene could rescue early as well as late developmental defects.

We obtained profilin III specific polyclonal antibodies using recombinant protein for immunization. This allowed us to quantify the profilin III concentrations in wild type and in the reverted pI/II-minus mutant cells. Whole cell homogenates from wild type (Fig. 3B) and the spontaneous revertant were separated

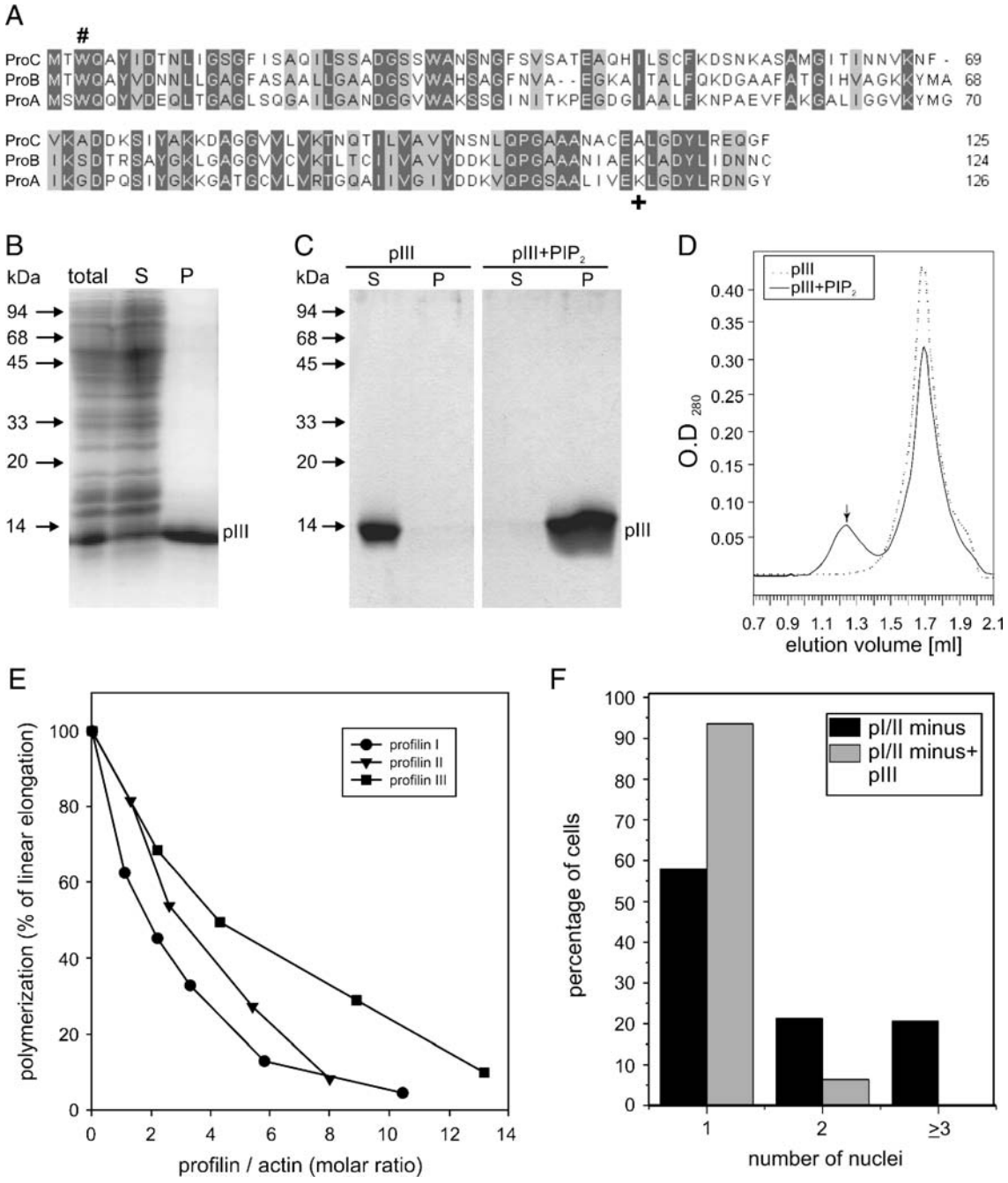



Fig. 2. Sequence comparison and biochemical analysis of profilin III. (A) An alignment of the profilin III (*proC*, DDB0215352) protein sequence with the profilin I (*proA*, DDB0191178) and II (*proB*, DDB0191249) isoforms shows the usual limited homology among profilins. Identical residues are marked with a dark, homologous residues with a grey background. In *D. discoideum* profilin II the tryptophane residue at position 3 (#) was essential for poly-L-proline binding, the lysine close to the C-terminus (+) carried some importance for the interaction with actin and is not present in profilin III [27]. (B) Recombinant profilin III was solubilized from inclusion bodies (total), pulled down with poly-L-proline beads (S = supernatant; P = pellet), and analyzed by SDS-PAGE and Coomassie blue staining. Nearly all of profilin III bound to poly-L-proline. (C/D) The interaction of profilin III with $PIP_2$ was assayed by cosedimentation of the protein in the absence (C, left panel) or presence (C, right panel) of lipid vesicles, and by gel filtration on a Smart system (D, dotted line: profilin alone, solid line: profilin in the presence of $PIP_2$ vesicles). (E) All recombinant profilins have comparable inhibitory effects on actin polymerization. G-actin aliquots (3 μM, containing 10% pyrene-labeled actin) were incubated with increasing concentrations of the appropriate profilin isoform and in each experiment the inhibition of the rate of filament elongation was compared to the linear phase of polymerization (set to 100%) of actin alone. (F) Profilin III rescues the cytokinesis defect of the profilin I/II minus mutant. Whereas many null mutant cells contained two and more nuclei (black bars), the numbers were shifted back to normal if the mutant expressed profilin III after transformation with the appropriate vector.

by SDS-PAGE, transferred to nitrocellulose and analyzed with affinity purified polyclonal antibodies relative to a dilution series of recombinant protein which was loaded on the same gels. The signals on the immunoblot were quantified by ImageJ software and compared to published data on profilin I and II concentrations in *D. discoideum* [23]. Remarkably, AX2 wild type cells expressed about 200 fold less profilin III (~0.23 μM) than profilin I and II (together ~47 μM). In the spontaneous pI/II

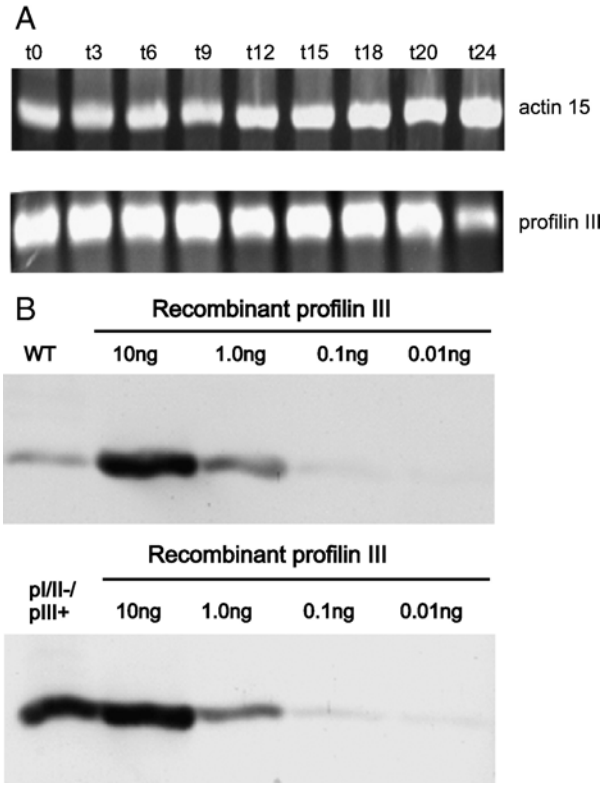



Fig. 3. Expression of *proC* during development and protein concentration. (A) In RT-PCR experiments total RNA was isolated after the onset of starvation between 0 and 24 h of development and used as a template for profilin III specific primers (bottom). Actin was used as a control (top). (B) The concentration of profilin III in wild type cells (top) or the spontaneous overexpressor in profilin I/II minus cells (bottom) was analyzed in immunoblots using the profilin III specific polyclonal antibody. A dilution series of recombinant profilin on the same gel was used for quantification.

revertant profilin III was about 30-fold upregulated. The relative abundance of the profilins was also examined by determining the mRNA levels for the three profilins through quantification by real-time RT-PCR experiments. In RNA from growing cells we obtained in a representative experiment 36.16 pg mRNA for profilin I, 21.39 pg for profilin II and 0.155 pg for profilin III, thus showing a 200-fold lower level for profilin III as compared to profilin I. These results agree very well with the ones obtained for the protein levels.

### *3.4. Subcellular distribution of profilin III and interaction with DdVASP*

Profilin I and II are distributed throughout the cell with some cortical enrichment [23,27]. The low abundance of profilin III rendered it impossible to analyze subcellular distribution by immunofluorescence in more detail. As expected from biochemical data, profilin III seemed to be localized in the cytoplasm, and wild type cells were only faintly stained. In earlier experiments we found that labeling of profilins with the rather large GFP moiety led to an inactive fusion protein which did not rescue the pI/II-minus phenotype. Therefore, profilin III was N-terminally fused to a Flag tag and transformed into wild type and pI/pII-minus mutants. As a control, an untagged profilin III was expressed in these strains as well. Profilin III was also present throughout the cells and showed some cortical enrichment. However, most interesting was the staining of filopodia, mainly in the tips (Fig. 4A). Among the filopodial proteins which could be targets for *D. discoideum* profilins are the formin dDia2 and the vasodilator-stimulated phosphoprotein DdVASP. As reported, dDia2 was found to interact specifically with profilin II, and not with profilin I and III [31]. The other candidate for interaction was DdVASP which contains an internal proline-rich domain as well. Both dDia2 as well as DdVASP are accumulated at filopodial tips [31,42–44]. In a yeast-two-hybrid approach we found that profilin III, in contrast to profilins I and II interacted with DdVASP (Fig. 4B). A detailed domain analysis points to the proline-rich region as being required for this interaction. The full length protein, the domains EVH1-PRD, and PRD-EVH2 show an interaction with profilin III, whereas the domain EVH2 or the PRD only did not (Fig. 4C).

### *3.5. Isolation of a profilin III null mutant*

A profilin III mutant was generated by homologous recombination in the wild type strain AX2. For this purpose, a gene replacement vector containing fragments of 400 bp and 600 bp up- and downstream of the *proC* gene was generated and transformed into AX2 cells. In knockout mutants the *proC* coding region should be replaced by the blasticidin resistance cassette. DNA from resistant clones was subjected to PCR analysis as depicted in Fig. 5 and the absence of profilin III was confirmed in Western blots. Rescue mutants were generated by expressing full length profilin III tagged with a Flag peptide at its N-terminus (Fig. 5).

In contrast to the pI/pII-minus mutants, removal of profilin III had no drastic effects on cytokinesis and development. There was, however, a clear defect in cell polarization during directed movement in a chemotaxis assay towards a microcapillary filled with the chemoattractant cAMP. As shown in Fig. 6, wild type cells move highly oriented along the chemotactic gradient and extend only a few pseudopods away from the main track. This is completely different with the profilin III knockout cells. The cells show a directed movement, but they are significantly slower, less polarized and develop more sideward pseudopods. This behaviour can be rescued by re-expression of profilin III.

## 4. Discussion

Profilins are considered to be abundant proteins that are involved in actin-filament dynamics in a cell as they can promote filament assembly presumably by catalysing the ATP-ADP-exchange on G-actin and are also involved in F-actin depolymerisation [22]. However, profilins are not only interacting with actin. Through their binding to phospholipids and to proteins that harbor proline-rich sequences they may also be components of signal pathways to the cytoskeleton [45]. Genetic analysis in yeast showed that a loss of the single profilin gene goes along with changes in the actin distribution [46], in *Drosophila* a loss of profilin leads to impaired oocyte development [47], whereas overexpression mimics mutations in the capping

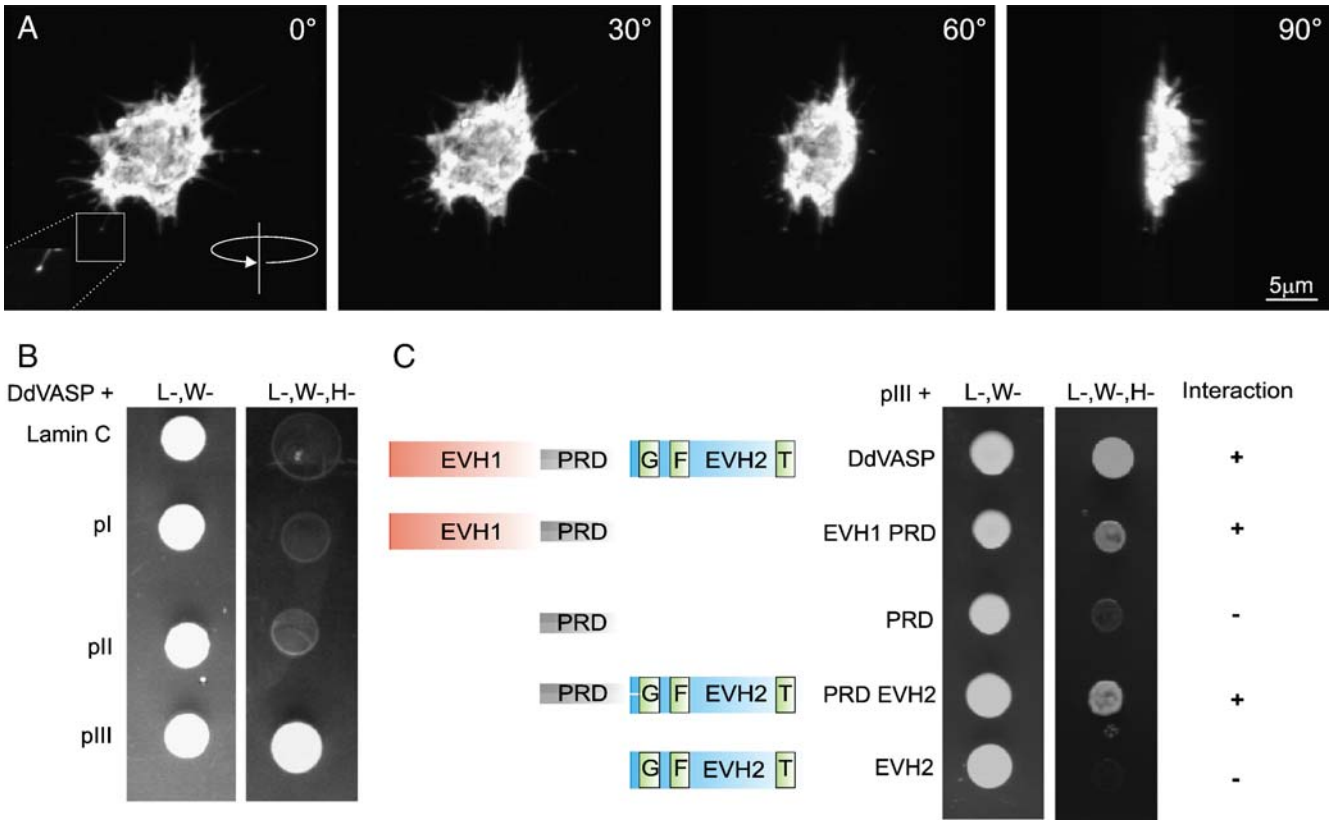



Fig. 4. Profilin III localizes to filopodia and interacts with DdVASP. (A) Profilin I/II- minus cells that overexpressed profilin III were fixed, stained with profilin III specific polyclonal antibodies and imaged in a confocal microscope. The three-dimensional reconstructions were turned in 30° intervals and for better visualization an enlarged segment of the cell is shown as an insert (left panel). (B) DdVASP interacts specifically with profilin III in a yeast-two-hybrid assay as tested for growth on selective media lacking leucine (L), tryptophane (W) or histidine (H). (C) Yeast cells were transformed with profilin III and the indicated DdVASP constructs fused to the activation domain of the yeast transcription factor GAL4. Both EVH1 and EVH2 domains that contained in addition the proline-rich domain (PRD) interacted with profilin III as indicated by the growth on the selective media.

protein [48]. In *D. discoideum* lack of either profilin I or profilin II, which are 55% identical and have very similar characteristics, does not lead to obvious changes. However, double mutants display severe defects in cytokinesis, pinocytosis, motility, and development and showed an increased phagocytosis rate [23,49]. In support of a role in actin sequestration we observed in the double mutant an increased F-actin concentration. A search for suppressors of the developmental phenotype identified a homologue of the mammalian CD36/LIMP-II [50,51]. As there is no direct interaction between profilin and the CD36 homologue an altered phosphoinositide-based signaling might explain the suppression. In *A. thaliana* profilin overexpression stimulates root hair growth, whereas antisense RNA expression leads to reduced cell elongation and developmental alterations [52,53].

*D. discoideum* contains an almost complete set of proteins which interact with monomeric actin [5]. One should keep in mind however, that an equimolar interaction between G-actin and its binding protein does not necessarily lead to an actin sequestering function. A thoroughly described example is the G-actin binding activity of CAP, the cyclase associated protein, not to be confused with filament capping proteins like CapG or Cap32/34. The cyclase associated protein is associated with the cell cortex, it is not abundant and its interaction with G-actin certainly does not change the overall G-/F-actin equilibrium which by rule of thumb is about 50% of total actin in the unpolymerized pool [54]. Therefore, it cannot be classified as a G-actin sequestering protein and it remains to be shown whether its interaction with actin just triggers enzymatic reactions or changes the structure of the complex using the actin molecule as a cytoskeleton-independent subunit.

The profilin data as presented in this study point into a very similar direction. *D. discoideum* profilin III is not abundant, it represents only about 0.5% of total profilin. It has the potential to function as a sequestering protein because it contains all the activities which are typical for a regular profilin. This includes its interaction with G-actin, poly-L-proline, phosphoinositides, and consequently the inhibition of actin polymerization. Even in vivo it exhibits these activities because a spontaneous overexpression of the wild type gene or a forced overexpression after transformation with the appropriate vector rescues the heavy defects of the profilin I/II minus mutants. But how could one profilin III out of 200 profilin I and II molecules supply any significant input towards sequestration of G-actin? We have to get used to the working hypothesis that actin is only at a first glance a cytoskeletal protein but that it might also function as a subunit of larger complexes such as RNA polymerases [55], completely independent of the actin cytoskeleton, and that bona

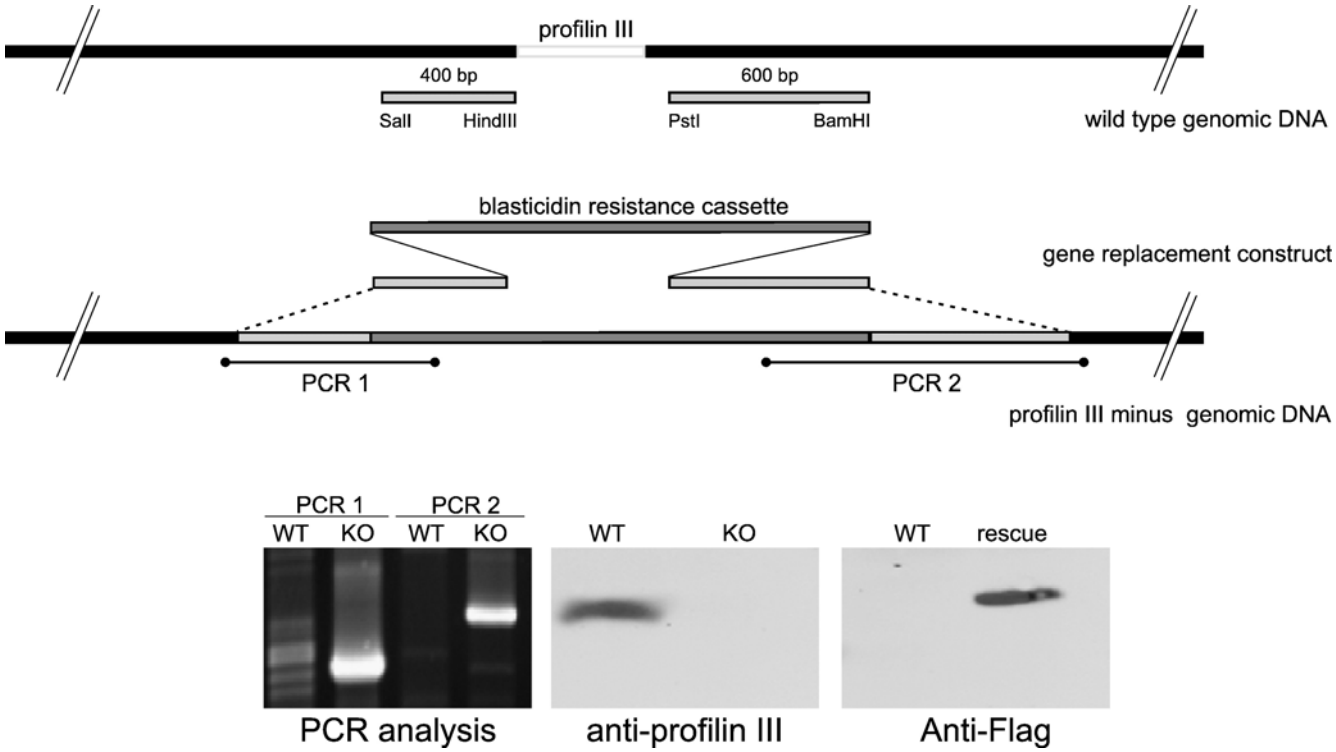
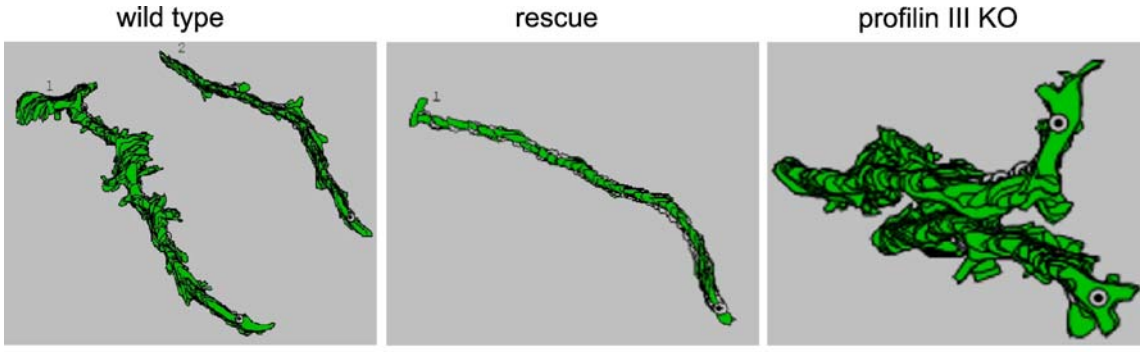



Fig. 5. Isolation of *proC* null and N-Flag-profilin III rescue mutants. Top: schematic representation of the linearized targeting construct used to inactivate the profilin III gene in AX2 wild type cells. The 5′ and 3′ homologous regions used originate from genomic DNA upstream and downstream of the profilin III coding region. Bottom: for identification of profilin III null mutants (left panel) a PCR screen was done with primers from the blasticidin cassette and regions outside of the gene replacement construct. Products in the PCR1 and PCR2 experiments showed that the desired gene disruption had occurred by replacing the complete coding region with the resistance cassette. The absence of profilin III was confirmed in an immunoblot using profilin III specific polyclonal antibody (middle panel). The profilin I/II minus mutant was rescued by the expression of an N-Flag-tagged profilin III; a homogenate from wild type cells was used as a control for the specificity of the anti-Flag tag antibody (right panel).




| | Speed [μm/min] | Persistence [μm/min-deg] |
|---|---|---|
| wild type | 13.2+/- 0.5 | 4.4+/- 0.1 |
| pIII rescue | 18.2+/- 7.1 | 8.7+/- 4.9 |
| pIII KO | 5.8+/- 5.4 | 1.7+/-2.0 |

Fig. 6. Single cell movement during chemotaxis. Cells were developed in phosphate buffer for 7 h, plated on a glass coverslip and chemotactically stimulated by a micropipette filled with $10^{-4}$ M cAMP. Cell movement was recorded at 6 frames per minute and analyzed using the DIAS software program (Soll Tech., USA). Representative cells from wild type (left), profilin III minus (right), and profilin III minus cells rescued with N-Flag-tagged profilin III (middle) are shown. The numbers average the data from about 30 cells each and indicate that a removal of profilin III leads to slower and less polarized cell movement.

fide actin binding proteins perhaps never see actin because they are bound to other target proteins. Consistent with this suggestion is the apparently highly specific interaction of DdVASP with profilin III despite the structural and functional similarity with the other two isoforms profilin I and II.

A peculiar feature of the profilin III knockout mutant is the reduced cell speed and polarization in a chemotaxis gradient. The extremely low abundance of profilin III cannot trigger significant cytoskeletal rearrangements just by formation of profilin:actin complexes or by an inefficient shuttling of actin to mostly capped barbed filament ends. Thus, a change in directionality during cell migration and in formation of pseudopods at the moving front after disruption of the profilin III gene most likely is caused by a finely tuned signaling activity via a profilin III binding protein like e.g. DdVASP. It is intriguing that a very similar phenotype was observed in the CAP (cyclase associated protein) minus mutant [56]. As mentioned above, this protein has G-actin and $PIP_2$ binding activity, and it contains a stretch with an increased number of proline residues. In addition, the chemoattractant-induced cGMP production is lowered and a direct interaction with the adenylate cyclase would be able to regulate directed cell motility. It is tempting to assume that an interaction between profilin III and this type of regulatory protein might relay a large signal to migration behaviour, completely independent of a hypothetical sequestering activity in vivo.

If one compares all profilin sequences that are currently available (see Fig. 1) one cannot speculate about specific and cytoskeleton-independent functions. In this phylogenetic tree the *D. discoideum* profilins I, II and III are neighbours and reflect only the potential of profilin III to function like the other two isoforms. This suggests that a phylogenetic comparison should not be subject to over-interpretation and that biochemical or cell biological data are necessary to increase the resolution of such a tree. It is intriguing however, that two profilin subfamilies originate very early from one ancestor and that the two emerging groups contain either only vertebrate and viral profilins or profilins from all other organisms including profilin 4 from vertebrates. At first it is surprising that viral and vertebrate profilins are in the same group, but one should take into account that the only evolutionary pressure for a viral profilin exists in the host cell of the vertebrate and consequently follows the changes of the vertebrate's profilins.

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